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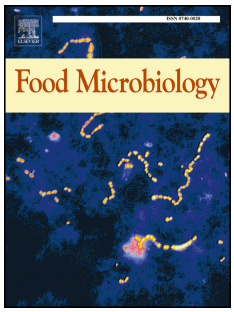

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Unveiling *hákarl*: a study of the microbiota of the traditional Icelandic fermented fish

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Abstract

Hákarl is produced by curing of the Greenland shark (*Somniosus microcephalus*) flesh, which before fermentation is toxic due to the high content of trimethylamine (TMA) or trimethylamine N-oxide (TMAO). Despite its long history of consumption, little knowledge is available on the microbial consortia involved in the fermentation of this fish. In the present study, a polyphasic approach based on both culturing and DNA-based techniques was adopted to gain insight into the microbial species present in ready-to-eat *hákarl*. To this aim, samples of ready-to-eat *hákarl* were subjected to viable counting on different selective growth media. The DNA directly extracted from the samples was further subjected to Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and 16S amplicon-based sequencing. Moreover, the presence of Shiga toxin-producing *Escherichia coli* (STEC) and *Pseudomonas aeruginosa* was assessed via qualitative real-time PCR assays. pH values measured in the analyzed samples ranged from between 8.07±0.06 and 8.76±0.00. Viable counts revealed the presence of total mesophilic aerobes, lactic acid bacteria and Pseudomonadaceae. Regarding bacteria, PCR-DGGE analysis highlighted the dominance of close relatives of *Tissierella creatinophila*. For amplicon sequencing, the main operational taxonomic units (OTUs) shared among the data set were *Tissierella*, *Pseudomonas*, *Oceanobacillus*, *Abyssivirga* and *Lactococcus*. The presence of *Pseudomonas* in the analyzed samples supports the hypothesis of a possible role of this microorganism on the detoxification of shark meat from TMAO or TMA during fermentation. Several minor OTUs (<1%) were also detected, including *Alkalibacterium*, *Staphylococcus*, *Proteiniclasticum*, *Acinetobacter*, *Erysipelothrix*, *Anaerobacillus*, *Ochrobactrum*, *Listeria* and *Photobacterium*. Analysis of the yeast and filamentous fungi community composition by PCR-DGGE revealed the presence of close relatives of *Candida tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. zeylanoides*, *Saccharomyces cerevisiae*, *Debaryomyces*, *Torulaspora*, *Yamadazyma*, *Sporobolomyces*, *Alternaria*, *Cladosporium tenuissimum*, *Moristroma quercinum* and *Phoma/Epicoccum*, and some of these species probably play key roles in the development of the sensory qualities of the end product. Finally, qualitative real-time PCR assays revealed the absence of STEC and *Pseudomonas aeruginosa* in all of the analyzed samples.

Keywords: *Tissierella*; *Pseudomonas*; *Debaryomyces*; 16S amplicon-based sequencing; PCR-DGGE.

1. Introduction

Fermentation represents one of the most ancient techniques for food preservation. Traces of this practice can be seen as far back as 6000 B.C. in the Fertile Crescent (Franco et al. 2016). Moreover, the production of fermented foods became very popular among the Egyptian, Greek and Roman civilizations (Huang, 2016).

Food fermentation is mainly based on the metabolic activities of microorganisms that are either naturally present in the raw materials or artificially inoculated (Shiferaw Terefe, 2016). The most well-known processes include lactic acid fermentation, fungal fermentation, and alkaline fermentation, where pro-technological microorganisms improve the aroma, flavor, texture, and nutritional characteristics of the raw materials and inhibit spoilage and pathogenic microorganisms (Shiferaw Terefe, 2016). Moreover, species belonging to some microbial groups mostly associated with food fermentation (e.g., lactic acid bacteria) can reduce the health hazards associated with the consumption of food containing some toxic substances (Luz et al., 2018). In this context, microorganisms can be considered human beings' coevolutionary partners responsible for providing a wide variety of fermented foods with enhanced nutritional and sensory characteristics.

Most fermented foods available on the market are still produced in accordance with ancient traditions deeply rooted in the territory of origin. The obtained products represent an invaluable source of microbial diversity where complex microbial populations coexist in a dynamic equilibrium.

The most popular fermented foods are produced with raw materials from the dairy, meat or vegetable food chains. For such products, an ample scientific literature on both manufacturing technologies and the microbial communities involved during their transformation is available. Regarding fermented foods produced with raw materials from the marine environment, a lack of knowledge on the technological processes and the relevant microbial populations is highlighted (Rajauria et al., 2016). Notwithstanding, fermented marine-based products are currently consumed by several cultural groups worldwide (Rajauria et al., 2016).

Brilliant examples of traditional fermented fish products are represented by *surströomming* and *rakfisk*, produced in Sweden and Norway, respectively, and *hákarl*, produced in Iceland (Skåra et al., 2015). The production of such delicacies dates back to the Viking Age, when preservation of foodstuffs with salt was expensive, especially in the remote regions of northern Europe. Therefore, instead of salting, new empiric methods of preservation of caught fish were carried out by local populations, thus leading to the production of edible and safe products.

Among the abovementioned fermented fish, *hákarl* is produced by curing of the Greenland shark (*Somniosus microcephalus*). As reported by Skåra et al. (2015), the origin of the production technique of *hákarl* is still not clear, and it is unknown whether the shark was specifically caught or simply collected from specimens that drifted ashore.

The consumption of fresh Greenland shark is considered unsafe, although the toxic substances responsible of poisoning have not been recognized. Different authors have reported cases of poisoning from the flesh of the Greenland shark likely due to a high level of trimethylamine (TMA) (Anthoni et al., 1991; Halsted, 1962; Simidu, 1961).

In ancient times, *hákarl* was produced by cutting the shark into pieces that were left to ferment for weeks or months in gravel pits often close to the sea. The pits were usually covered with stones, seaweed, or turf. These structures were constantly exposed to seawater, which flooded over the fish at high tide (Skåra et al., 2015).

In the modern era, the fermentation of shark pieces is carried out in closed containers that allow the resulting leachate to be drained. Such a process can last from 3 to 6 weeks depending on the environmental temperature and season. After fermentation, the shark pieces are further cut and hung to dry in dedicated sheds for weeks or months, depending on the outdoor environmental conditions (Skåra et al., 2015).

In both the ancient or the modern processes, the metabolic activities of microorganisms occurring during shark fermentation lead to the conversion of a poisonous raw material into a safe and tasty ready-to-eat food product with a long shelf-life. The *hákarl* is characterized by a soft texture with a whitish cheese-like appearance, strong ammonia smell and fishy taste (Skåra et al., 2015).

Despite the long history of *hákarl* consumption, a lack of knowledge is available on the microbial consortia involved in the shark fermentation. Indeed, to our knowledge, only one study that dates back to 1984 is available in the scientific literature (Magnússon and Gudbjörnsdottir, 1984).

Since many years ago, the cultivation of microorganisms on synthetic growth media was the primary way to study microbial communities in foods. The development of molecular techniques based on the use polymerase chain reaction (PCR) opened new frontiers for the study of microbial ecology in complex matrices (Garofalo et al., 2017). A variety of studies have shown that combinations of different microbiological techniques can provide sound information on the microbial composition of complex food matrices, including those subjected to fermentation. Among the most adopted and sensitive molecular methods, PCR-Denaturing Gradient Gel Electrophoresis (DGGE), real-time PCR and next-generation sequencing provide reliable data for microbiological profiling of foods.

Based on these concepts, a polyphasic approach based on both culture and DNA-based techniques was adopted to provide insight into the microbial species present in ready-to-eat *hákarl*.

To this end, samples of ready-to-eat *hákarl* were subjected to viable counting on different selective growth media. The DNA directly extracted from the samples was further subjected to PCR-DGGE and Illumina sequencing. Moreover, the presence of *Shiga* toxin-producing *E. coli* (STEC) and *Pseudomonas aeruginosa* was assessed via qualitative real-time PCR assays.

2. Materials and methods

2.1. Sampling

Ten samples of ready-to-eat *hákarl* (Figure 1) codified from H1 to H10 were analyzed, for each sample two 100 g boxes were purchased (for a total of 20 analyzed boxes). The samples were purchased via the internet from a dealer located in Iceland. In more detail, the samples were collected through different orders placed from January to May 2018. The samples were shipped by international express courier in plastic boxes at room temperature in aerobic conditions and analyzed after 24 hours from shipping. No further information on the samples was provided by the producer.

2.2. pH measurements

pH values of the *hákarl* samples were determined with a pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy). For each sample, the measurements were performed in duplicate.

2.3. Microbial viable counts

Twenty-five grams of each *hákarl* sample were homogenized for 5 min at 260 rpm in 225 mL of sterile peptone water (bacteriological peptone 1 g L^{-1}, Oxoid, Basingstoke, UK) using a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy). The obtained suspensions were diluted 10-fold and subjected to microbial counts of total mesophilic aerobes, lactic acid bacteria, Pseudomonadaceae, Enterobacteriaceae and eumycetes. Briefly, total mesophilic aerobes were counted as reported by Osimani et al. (2011); presumptive mesophilic lactobacilli and lactococci were enumerated in De Man, Rogosa and Sharpe (MRS) agar medium incubated at 30 °C for 48 h and M17 agar medium incubated at 22 °C for 48 h, respectively as previously described (Aquilanti et al., 2013). The enumeration of Pseudomonadaceae was carried out using Pseudomonas Agar Base (PAB) with cetrimide-fucidin-cephalosporin (CFC) selective supplement (VWR International, Milan, Italy), incubated at 30 °C for 24–48 h (Garofalo et al., 2017), whereas Enterobacteriaceae were counted in Violet Red Bile Glucose Agar (VRBGA) incubated at 37 °C for 24 h (Garofalo et al., 2017). Finally, the enumeration of eumycetes was carried out on Wallerstein Laboratory Nutrient (WLN) agar medium supplemented with chloramphenicol (0.1 g/L) to inhibit the growth of bacteria and incubated at 25 °C for 72 h (Taccari et al., 2016).

A miniVIDAS apparatus (Biomerieux, Marcy l'Etoile, France) was used to assess the presence of *Listeria monocytogenes* through the enzyme-linked fluorescent assay (ELFA) method in accordance with the AFNOR BIO 12/11-03/04 validated protocol (Aquilanti et al., 2007).

2.4. DNA extraction from hákarl samples

Aliquots (1.5 mL) of each homogenate (dilution 10^{-1}) prepared as described above were centrifuged for 5 min at 16000 g, and the supernatants were discarded. The cell pellets were then used for the extraction of total microbial DNA using an E.Z.N.A. soil DNA kit (Omega bio-tek, Norcross, GA, USA) following the manufacturer's instructions. A Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) was used to measure the quantity and purity of the extracted DNAs, which were then standardized to a concentration of 25 ng μL^{-1} for further analysis. DNA extracts obtained from the *hákarl* from each of the two boxes representing one sample (H1-H10) were then pooled and subjected to *PCR-DGGE* analyses and 16S rRNA gene amplicon target sequencing (Milanović et al., 2018).

2.5. PCR-DGGE analysis of bacteria

The extracted DNA was first amplified by PCR in a My Cycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the universal prokaryotic primers 27F and 1495R described by Weisburg et al. (1991) for the amplification of 16S rRNA gene. In detail, 2 µL (approximately 50 ng) of DNA from each sample was amplified in a 25 µL reaction volume composed of 0.5 U of Taq DNA polymerase (Sibenzyme, Novosibirsk, Russia), 1X reaction buffer, 0.2 mM dNTPs and 0.2 µM of each primer using the cycling program described by Osimani et al. (2015). The PCR products were checked by routine electrophoresis on 1.5% agarose (w/v) gels and then purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) according to manufacturer's instructions. 2 µL of the purified PCR products was used as a template for the amplification of the V3 region of the 16S rRNA gene with the 338F-518R primer pair (Alessandria et al., 2010). The forward primer, 338F, was attached with the GC clamp necessary for the following DGGE analysis as described by Ampe et al. (1999). The PCR conditions were those described by Osimani et al. (2015), except for the Taq polymerase (Sibenzyme) used in the present study. 5 µL of PCR amplicons was loaded on a 1.5% agarose (w/v) gel with a 100 bp molecular weight marker (HyperLadder™ 100 bp) to check for the expected PCR product size of 180 bp prior to the PCR-DGGE analysis. Subsequently, 20 µL of the PCR products was loaded on a 30-60% urea-formamide (w/v) gradient DGGE gel (100% corresponds to 7 M urea and 40% (w/v) formamide), and the gel was run at a constant voltage of 130 V for 4 h at 60 °C in 1× TAE buffer (0.04 mol

L^{-1} Tris–acetate, 0.001 mol L^{-1} EDTA) in a DGGE Bio-Rad D-code™ apparatus (Bio-Rad Laboratories). After the DGGE run, the gel was stained with SYBR Green I Stain 1X (Lonza, Walkersville, MD, USA) in 1X TAE for 30 min, visualized under UV light and photographed with a Complete Photo XT101 system (Explera). All of the single bands visible by eye after UV light exposure were excised with gel cutting pipette tips, introduced into 50 µL of molecular biology grade water and left overnight at 4 °C to allow the elution of the DNA. 5 µL of the eluted DNA was amplified by PCR as described above but using the 338F and 518R primers without the GC clamp. The amplicons were checked by electrophoresis and sent to Genewiz (Takeley, UK) for purification and sequencing. The resulting sequences in FASTA format were compared with those previously deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), and only the sequences showing ≥ 97% similarity was unambiguously assigned into species or genus levels.

2.6. PCR-DGGE analysis of the yeast and filamentous fungal communities

DNA extracted as previously reported was used for the analysis of the yeast and filamentous fungal communities. An approximately 250 bp long fragment of the D1/D2 region of the 26S rRNA gene was amplified using NL1 (5'-GCC ATA TCA ATA AGC GGA GGA AAA G-3') and LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') primers (Cocolin et al., 2000). An additional GC clamp (5'-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG G-3') was added to the NL1 forward primer. The amplification reactions and conditions were carried out as described in Palla et al. (2017). The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) agarose gels stained with 20,000X REALSAFE Nucleic Acid Staining Solution (Durviz, s.l., Valencia, Spain). All gels were visualized using UV light and captured as TIFF format files using the UVI 1D v. 16.11a program for the FIRE READER V4 gel documentation system (Uvitec Cambridge, Eppendorf, Milan, Italy).

The amplicons were analyzed using the DCode™ Universal Mutation Detection System (Bio-Rad, Milan, Italy). Twenty µL of the PCR products in 20 µL of a 2x buffer consisting of 70% glycerol, 0.05% xylene cyanol and 0.05% bromophenol blue were loaded on an 8% polyacrylamide-bisacrilamide (37.5:1) gel with a urea-formamide denaturing gradient ranging from 20% to 80%. The gels were run at 80 V and 60 °C for 16 hours and stained for 30 min in 500 mL of 1x TAE buffer containing 50 µL of Sybr® Gold Nucleic Acid Gel Stain (Life Technologies, Milan, Italy). The profiles were visualized as previously described. The bands of interest in the DGGE profiles were cut out from the gels for sequencing. DNA was extracted by eluting for 3 days in 50 µL 10 mM TE at 4 °C. One µL of the supernatant diluted 1:100 was used to reamplify the D1/D2 regions of the DNA according to the PCR protocol described above using an NL1 primer without the GC clamp. The amplification products were then purified with the UltraClean PCR

CleanUp Kit (MO-BIO Laboratories, CABRU Sas, Arcore, Italy) according to the protocol of the manufacturer, quantified and 5' sequenced at the Eurofins Genomics MWG Operon (Ebersberg, Germany). Sequences were analyzed using BLAST on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The related sequences were collected and aligned using MUSCLE (Edgar, 2004a, b), and phylogenetic trees were constructed using the maximum likelihood method based on the Kimura 2-parameter model (Kimura, 1980) using Mega 6 software (http://www.megasoftware.net/) with 1000 bootstrap replicates (Tamura et al., 2013). The sequences were submitted to the European Nucleotide Archive under the accession numbers from LS990841 to LS990863.

2.7. 16S rRNA gene amplicon target sequencing

DNA directly extracted from *hákarl* samples was quantified using a QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized at 20 ng μL^{-1} and used a template in the PCR amplifying the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al. (2013).

The PCR amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy), and the resulting products were tagged using the Nextera XT Index Kit (Illumina Inc. San Diego. CA) according to the manufacturer's instructions. After the second clean-up, the amplicons were quantified using a QUBIT dsDNA Assay kit and equimolar amounts of the amplicons from different samples were pooled. The pooled samples were analyzed with an Experion workstation (Biorad, Milan, Italy) for quality analysis prior to sequencing. The sample pool was denatured with 0.2 N NaOH, diluted to 12 pM, and combined with 20% (vol/vol) denatured 12 pM PhiX prepared according to Illumina guidelines. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry to generate 250 bp paired-end reads according to the manufacturer's instructions.

2.7.1. Bioinformatics analysis

After sequencing, the paired-end reads were first joined using FLASH software (Magoc and Salzberg, 2011) with default parameters. Joint reads were quality filtered (at Phred < Q20) using QIIME 1.9.0 software (Caporaso et al., 2010) and the pipeline recently described (Ferrocino et al., 2017). Briefly, USEARCH software version 8.1 (Edgar et al., 2011) was used for chimera filtering and Operational Taxonomic Units (OTUs) were clustered at a 99% similarity threshold using UCLUST algorithms (Edgar, 2010). Centroid sequences of each cluster were mapped against the Greengenes 16S rRNA gene database version 2013 for taxonomic assignment. To avoid biases due to different sequencing depths, OTU tables were rarefied at 11010 sequences. The OTU table displays the higher taxonomy

resolution that was reached, and the two biological replicates from each sampling point were averaged. The tables were then imported in the Gephi software (Bastian et al., 2009), and an OTU network was built.

All of the sequencing data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (SRA accession number: SRP153795).

2.7.2. Statistical analysis

Statistics and plotting were carried out in the R environment (www.r-project.org). Alpha diversity indices were calculated using the *diversity* function of the vegan package (Dixon, 2003). Weighted and unweight UniFrac distance matrices and OTUs table were used to find differences between the samples using Anosim and Adonis statistical tests through the function *vegan* in the R environment. Pairwise Wilcoxon tests were used, as appropriate, to determine significant differences in alpha diversity or OTU abundance. The principal component analysis was plotted using the function *dudi.pca* through the *made4* R package.

2.8. Real-time PCR analyses for the detection of foodborne pathogens

Real-time PCR analyses were performed on a RotorGene Q thermal cycler (Qiagen, Hiden, Germany) exploiting TaqMan chemistry. All target probes employed were dual-labeled with 5'-FAM and a 3'-nonfluorescent quencher (as specified below). The oligonucleotides were purchased from ThermoFisher Scientific (Milan, Italy) and from LCG Biosearch Technologies (Petaluma, CA, USA). The reaction mixtures were all prepared at a final 25 µl reaction volume. Molecular-grade H_2O was included in each analytical session as a negative control, as well as DNA from reference strains as positive controls. Fluorescence was measured in the green channel for the target genes, and in the yellow channel for the Internal Amplification Control.

2.8.1. Detection of Shiga-toxin E. coli (STEC)

STEC detection was performed according to the standard ISO/TS 13136:2012 specifications, as also previously reported (Petruzzelli et al. 2013). Briefly, this method initially targets the Shiga-toxin genes *stx1* and *stx2*, followed by the *eae* adhesion factor and serogroup-specific genes (O157, O145, O103, O111, O26 and O104:H4). Amplification of 2 µl of template DNA was performed using the QuantiFast Pathogen PCR+IC kit (Qiagen) in combination with the

previously reported primer set and 5'-FAM-3'-MGBNFQ dual-labeled probes (Osimani et al. 2018). An Internal control DNA and Internal Control Assay to be added to the reaction mix were provided with the kit.

DNA from STEC strains provided by the EU Reference Laboratory for STEC – Istituto Superiore di Sanità (Rome, Italy) were included as positive controls.

2.8.2. Detection of Pseudomonas aeruginosa

P. aeruginosa was detected using the QuantiFast Pathogen PCR+IC kit together with a primer-probe set identifying the presence of the *ecfX* gene, which encodes an extracytoplasmic sigma factor. The probe was dual-labeled with 5'-FAM and 3'-BHQ1, and the assay mixture was prepared as described by Amagliani et al. (2013). Amplification of 2 µl of template DNA was performed following the thermal protocol indicated by the same authors. DNA from a previously prepared boiled extract of *P. aeruginosa* ATCC 27853 was included as a positive amplification control in this assay.

3. Results

3.1. pH measurements and microbial viable counts

The pH values measured in the analyzed samples ranged from between 8.07±0.06 and 8.76±0.00 (Table 1). The results of the viable counts are reported in Table 1. In more detail, the mean values of the total mesophilic aerobes ranged from between 1.00±0.00 and 5.71±0.09 log cfu g^{-1}. Low counts of LAB on MRS at 30 °C were recorded with mean values from between < 1 and 1.60±0.43 log cfu g^{-1}. Regarding the LAB counted on M17 at 22 °C, the mean values ranged from between 1.95±0.07 and 4.51±0.04 log cfu g^{-1}. Pseudomonadaceae counts showed mean values from between <1 and 1.59±0.16 log cfu g^{-1}. For both the Enterobacteriaceae and Eumycetes counts, mean values <1 log cfu g^{-1} were recorded. Finally, no *Listeria monocytogenes* was detected.

3.2. PCR-DGGE analyses

3.2.1. Bacteria

Regarding the bacteria, the results of PCR-DGGE analysis of the *hákarl* samples are reported in Table 2, while Supplementary Figure 1 shows the DGGE profiles obtained from the analysis of the microbial DNA directly extracted from the samples.

In more detail, the dominance of close relatives to *Tissierella creatinophila* was clear in all of the pooled samples with sequence identities from between 85 and 98%. Moreover, close relatives to *Anaerosalibacter* species were detected in the pooled samples H1, H2 and H10. Finally, close relatives to *Murdochiella massiliensis*, *Sporanaerobacter acetigenes* and *Pontibacillus marinus* were also found in samples H5, H6 and H10, respectively.

3.2.2. Yeast and filamentous fungal communities

A DNA fragment of approximately 250 bp containing the partial D1/D2 domain of the 26S rRNA gene was successfully amplified from all of the samples, except H1. DGGE analysis of the PCR products showed distinctive patterns characterized by intense and clearly defined fragments (Supplementary Figure 2). With the goal of identifying the microbial species present in *hákarl*, the main DGGE bands were excised, sequenced and matched to species by using BLAST and phylogenetic trees analyses (Table 3 and Figure 2). The sequences matched the yeast species *Candida tropicalis, C. glabrata, C. parapsilosis, C. zeylanoides, Saccharomyces cerevisiae* and the yeast genera *Debaryomyces, Torulaspora, Yamadazyma, Sporobolomyces*. Figure 3 shows the relative percentages of the most abundant fungal genera detected in the different samples. For each sample, the percentage was calculated by dividing the number of fragments referring to a genus by their total number. Each *hákarl* sample showed a different yeast composition, with *Debaryomyces* occurring in all the samples, although at different levels. In three *hákarl* samples (H2, H6 and H7), sequences related to filamentous fungal species were also found. In particular, *Cladosporium tenuissimum* occurred in the H2 sample, while *Moristroma quercinum* was found in the H7 sample. The H6 sample was characterized by the presence of *Alternaria*, and genera belonging to the family Didymellaceae, such as *Phoma* and *Epicoccum*.

3.3. 16S rRNA gene amplicon target sequencing

The total number of paired sequences obtained from the 16S rRNA gene sequencing reached 3,208,571 raw reads. After merging, a total of 997.224 reads passed the filters applied through QIIME, with an average value of 49.861,2 ± 17.399,72 reads/sample, and a mean sequence length of 456 bp. The rarefaction analysis and Good's coverage, expressed as a median percentage (95%), also indicated satisfactory coverage for all samples (Supplementary Table 1). Alpha-diversity indicated a higher level of complexity and the highest number of OTUs when only taking into the account the sample H3 ($P < 0.05$). Adonis and analysis of similarity (ANOSIM) statistical tests based on weighted and unweighted UniFrac distance matrices showed significant differences among the samples ($P < 0.001$; $R = 0.980$). Differences between the samples were further demonstrated by principal-component analysis (PCA) based on the

335 relative abundance of the main OTUs (Fig. 4). The PCA clearly showed a separation between samples (ANOSIM
336 statistical test $P < 0.01$). As shown in Fig. 7, the main OTUs shared among the data set were *Tissierella* (78.6% of the
337 relative abundance) (Table 4), *Pseudomonas* (8.4%), *Abyssivirga* (4.0%), *Oceanobacillus* (6.7%) and *Lactococcus*
338 (0.2%), and based on the size of the edges, it was possible to see that *Tissierella* can be considered a core OTU of the
339 *hákarl* (Fig. 5 and Table 4). Several minor OTUs (<1%) were also detected, including *Listeria*, *Staphylococcus*,
340 *Photobacterium* and *Acinetobacter* (Table 4).
341
342 *3.4. Real-time PCR analyses*
343
344 Fluorescence signals resulting from real-time PCR assays were analyzed by manually positioning the cycle threshold at
345 the take-off point of the positive control's amplification curve relative to the gene under investigation.
346 All DNA samples analyzed yielded negative results for both pathogens of interest. Every reaction mixture, regardless of
347 the type of master mix or internal amplification control used, yielded positive signals in the yellow channel, thus
348 ensuring the absence of inhibition and excluding false negative results.
349 As for STEC strain, the first detection step (aimed at revealing the two Shiga toxin-encoding genes *stx1* and *stx2*) were
350 performed in singleplex, since the specific probes were labeled with the same fluorophore. All of the DNA samples
351 tested negative for both sequences; therefore, no further analysis of *eae* or serogroups was necessary according to the
352 ISO/TS 13136:2012. The reference strain used in this experiment was EURL-VTEC D07 *E. coli* O26 (stx1+, stx2+,
353 eae+).
354
355 **4. Discussion**
356
357 Among the fermented fish products of northern European countries, *hákarl* represents a masterful example of a delicacy
358 and niche product that, in former times, nourished the Icelandic populations (Skåra et al., 2015). It is noteworthy that
359 only a few producers are currently carrying out the production of *hákarl* in accordance with ancient traditions that
360 maintain the use of Greenland shark flesh.
361 Greenland shark is a slow growing, coldwater shark that can reach 21 feet in length. As reported by MacNeil et al.
362 (2012), the physiology of the Greenland shark is not generally well studied. It is noteworthy that high levels of
363 trimethylamine N-oxide (TMAO) have been detected in its flesh by different authors (Anthoni et al., 1991; Bedford et
364 al., 1998; Goldstein et al., 1967; Seibel and Walsh, 2002). The role of such a compound is not completely understood;
365 nevertheless, it is thought that the high concentrations found in polar fish suggest that this osmolyte may contribute to

366 the enhancement of osmotic concentrations, thus lowering the freezing point of the bodily fluids (MacNeil et al., 2012).
367 Moreover, both TMAO and its reduced form TMA represent low-density molecules that can increase the buoyancy of
368 the shark. It is also thought that, due to the high urea concentrations present in elasmobranchs like the Greenland shark,
369 TMAO might act as a counteracting solute that protects proteins from destabilization (MacNeil et al.,2012; Seibel and
370 Walsh, 2002).
371 The attention of the food industry and consumers towards locally produced traditional food is constantly increasing.
372 Although both the ancient and modern processing steps used to obtain *hákarl* from Greenland shark are mostly
373 recognized and standardized, less is known about the chemical and microbiological traits of such a food product. Based
374 on the physiology of the Greenland shark, it is thought that the microbiota occurring in *hákarl* might be strongly
375 influenced by the peculiarities of the flesh used as raw material. A 30-year-old study attempted to identify the bacterial
376 species in *hákarl* (Magnússon and Gudbjörnsdottir, 1984) with no mention of the possible occurrence of eumycetes.
377 Hence, to our knowledge, the microbiology of such a fermented food product has not yet been unveiled. The present
378 study aimed to reveal the microbial species occurring in ready-to-eat *hákarl* samples using a combination of traditional
379 microbiological culture-dependent (viable counts) and -independent methods (namely, PCR-DGGE, amplicon-based
380 sequencing and qualitative real-time PCR).
381 The samples under study were first subjected to pH measurements. The detected values were in agreement with those
382 reported by Skåra et al. (2015) for dried ready-to-eat *hákarl*. High pH values can be explained by microbial metabolic
383 activities that led to the conversion of urea, which is naturally present in Greenland shark flesh, into ammonia (Skåra et
384 al., 2015). Interestingly, Jang et al. (2017) reported similar pH values (8.4-8.9) in alkaline-fermented skate, which
385 represents a typical fermented seafood in South Korea. It is known that skates retain urea and trimethylamine N-oxide
386 in their muscles; hence, similarly to *hákarl*, the fermentation of skate, which is carried out at low temperature, leads to
387 the production of a distinctive odor due to the ammonia and trimethylamine produced during fermentation (Reynisson
388 et al., 2012).
389 In the present study, the viable counts of total mesophilic aerobes, presumptive mesophilic lactobacilli and lactococci,
390 Pseudomonadaceae, Enterbacteriaceae and eumycetes were assessed.
391 Regarding the total mesophilic aerobes, the counts were generally lower than the value of 8 log cfu g^{-1} reported by
392 Skåra et al. (2015) in *hákarl* at the end of ripening.
393 As far as the counts of lactic acid bacteria are concerned, little is known about the magnitude of their presence in
394 *hákarl*, though this microbial group is known to play a primary role in the production of many other traditional
395 fermented fish, such as *jeotgal* from Korea, *shidal* from India, rakfisk from Norway, and numerous fermented fish
396 products from China (Françoise, 2010; Majumdar et al., 2016; Skåra et al., 2015; Thapa et al., 2016; Xu et al., 2018;

Zang et al., 2018a). In addition to this, the occurrence of lactic acid bacteria in the marine environment has been reported (Gómez-Sala et al., 2016). Of note, the differences in the counts of presumptive lactococci in comparison with those of presumptive mesophilic lactobacilli in *hákarl*. This difference agrees well with the results from metagenomic sequencing that highlighted the sole presence of lactococci among lactic acid bacteria. Such a difference might tentatively be ascribed to both the higher adaptation of lactococci to shark flesh fermentation conditions and their acknowledged higher competitiveness in protein-rich substrates explained by their higher proteolytic and peptidase activities in respect with lactobacilli (Requena et al., 1993; Quigley et al., 2013; Terzic-Vidojevic et al., 2014), especially under alkaline conditions (Addi and Guessas, 2016).

The presence of Pseudomonadaceae has already been described in alkaline-fermented skate, where *Pseudomonas* was reported to be among the dominant genera (Jang et al., 2017; Reynisson et al., 2012).

Regarding Enterobacteriaceae, it is noteworthy that their minimum pH for growth is 3.8, with an upper limit of approximately 9.0; hence, even if they were present, the high pH of the samples did not allow Enterobacteriaceae to grow in the fermented flesh. Among Enterobacteriaceae, STEC strains represent a major health treat for humans due to their ability to produce food-borne infections (EFSA and ECDC, 2016). Although *E. coli* is not naturally harbored by fish, STEC strains have recently been isolated by Cardozo et al. (2018) from the fish *Nile tilapia*. Of note is the absence of STEC strains in all of the analyzed *hákarl* samples, as shown by real-time PCR assays.

In the present study, viable counts < 1 log cfu g^{-1} were reported for eumycetes.

The use of PCR-DGGE and amplicon sequencing allowed major and minor microbial species to be detected in the analyzed *hákarl* samples.

In more detail, a massive presence of *Tissierella* was detected by both PCR-DGGE and amplicon sequencing in all the analyzed samples. *Tissierella* belongs to the *Tissierellia* classis nov., which includes the genera *Anaerococcus*, *Anaerosphaera*, *Finegoldia*, *Gallicola*, *Helcococcus*, *Murdochiella*, *Parvimonas*, *Peptoniphilus*, *Soehngenia*, *Sporanaerobacter*, and *Tepidimicrobium*. Moreover, the misclassified species *Bacteroides coagulans* and *Clostridium ultunense* are also included.

Members of the class *Tissierellia* are Gram-positive or Gram-variable anaerobic obligate cocci and rods. As reported by Chen et al. (2018), the genus *Tissierella* comprises protein-degrading anaerobes that can produce volatile fatty acids such as acetic, butyric, and propionic acids. Moreover, it is acknowledged that *Tissierella creatinophila* grows on creatinine as sole source of carbon and energy (Harms et al., 1998a). *Tissierella* species can degrade creatinine via creatine, sarcosine, and glycine into monomethylamine, ammonia, carbon dioxide, and acetyl phosphate (Harms et al., 1998b). Interestingly, active *Tissierella* cells have already been reported among the major genera involved in the production of alkaline-fermented skate (Jang et al., 2017), thus confirming the possible adaptation of this microbial

genus to saline and alkaline conditions, as suggested by Jang et al. (2017). It is noteworthy that species belonging to the *Tissierella* genus have also been described as psychrotolerant microorganisms (Prevost et al., 2013), thus likely explaining the presence of members of such genus in the analyzed *hákarl* samples.

Regarding the presence of *Oceanobacillus*, which was detected only through amplicon sequencing, it is worth noting that this bacterial genus comprises Gram-positive, spore forming rods that are obligate aerobes, extremely halotolerant and facultatively alkaliphilic (Lu et al., 2001). Moreover, *Oceanobacillus* species can grow at temperatures between 15 and 42 °C and in pH range from 6.5 to 10, with an optimum pH between 7.0 and 9.5 (Lu et al., 2001). Kumar et al. (2012) demonstrated that halotolerant bacteria, including *Oceanobacillus*, can produce enzymes that are salt stable and active under extreme conditions. Interestingly, Yukimura et al. (2009) reported the isolation of *Oceanobacillus* strains from a glacial moraine in Qaanaaq, Greenland, and this finding likely explains the presence of these spore forming bacteria in the analyzed *hákarl* samples.

Amplicon sequencing supported the detection of *Abyssivirga*. This bacterial genus belongs to the Lachnospiraceae family and comprises strictly anaerobic, mesophilic and syntrophic organisms. To the our best knowledge, the sole species that belongs to this genus is represented by *Abyssivirga alkaniphila*, which is an alkane-degrading, anaerobic bacterium recently isolated from a deep-sea hydrothermal vent system (Catania et al., 2018; Schouw et al., 2016). This observation explains the presence of this bacterium in the marine environment and, hence, in the *hákarl* samples. This species can grow between 14–42 °C and within a pH range between 7.0 and 8.2. As reported by Schouw et al. (2016), who first described this species, *A. alkaniphila* can ferment carbohydrates, peptides and aliphatic hydrocarbons.

As for the presence of *Pseudomonas*, which was detected through amplicon sequencing, it is noteworthy that this bacterial genus has already been reported among the most abundant isolates in fermented skate (Jang et al., 2017). The genus *Pseudomonas* encompasses many alkaliphiles; among these, Yumoto al. (2001) reported the isolation of a novel facultatively psychrothrophic alkaliphilic species of *Pseudomonas*, being *Pseudomonas alcaliphila* sp. nov. This species of marine origin can grow at 4 °C and at pH 10 in the presence of NaCl; these features likely explain the presence of the genus *Pseudomonas* in the analyzed *hákarl* samples. Moreover, it is widely acknowledged that the *Pseudomonas* species responsible for fresh fish spoilage can also be present in fish-based foods (Stanborough et al., 2018). In this regard, Liffourrena et al. (2010) reported that some species of *Pseudomonas* possess TMA degradation pathways. More specifically, in *Pseudomonas aminovorans* TMAO is oxidized by a trimethylamine monooxygenase (TMA monooxygenase), whereas TMA can be directly dehydrogenated to formaldehyde and dimethylamine (DMA) by a trimethylamine dehydrogenase (TMA dehydrogenase). Similarly, in *Pseudomonas putida* the same enzymes, namely, TMA monooxygenase and TMA dehydrogenase, oxidize TMA under aerobic conditions (Liffourrena et al., 2010). On the one hand, these metabolic pathways represent a selective advantage for *Pseudomonas*, and on the other hand, they

lead to the removal of TMA (Liffourrena et al., 2010). Although the amounts of TMAO and TMA were not assessed in the present study, it can be hypothesized that the metabolic activity of *Pseudomonas* species could presumably lead to the detoxification of TMAO or TMA in the shark meat or during fermentation.

Of note, the pathogenic species *P. aeruginosa* was absent in all of the samples, as shown by qualitative real-time PCR assays.

In the analyzed *hákarl* samples, the presence of *Lactococcus* was also detected through amplicon sequencing. The presence of lactococci in the marine environment has already been demonstrated (Gómez-Sala et al., 2016); moreover, Alonso et al. (2018) recently reported the isolation of *Lactococcus* strains from the gut of marine fishes. As reported by Guan et al. (2011), lactic acid bacteria were among the dominant genera isolated from *saeu-jeot*, a Korean salted fermented food produced made with shrimp (*Acetes japonicas*). Although their role has not yet been fully clarified, lactococci were also detected in fermented skate and in *narezushi*, which is produced through the fermentation of salted fish with rice (Jang et al., 2017). As reported by Françoise (2010), lactic acid bacteria are generally less competitive in fish flesh than psychrotrophic Gram-negative bacteria; nevertheless, Ji et al. (2017) highlighted the essential role of lactic acid bacteria (including *Lactococcus*) in flavor definition during fish fermentation. It is also noteworthy that lactic acid bacteria can exert a potential biopreservative activity in seafood products (Ghanbari et al., 2013).

Regarding *Alkalibacterium*, the presence of this marine lactic acid bacteria has already been reported in marine environments and organisms (Jang et al., 2017) as well as in marine-based foods such as *jeotgal* (Guan et al., 2011). Interestingly, species belonging to the *Alkalibacterium* genus were found in fermented Spanish-style green table-olives and blue-veined raw milk cheese, thus confirming the high adaptation of this genus to halophilic and alkaline conditions (Lucena-Padrós et al., 2015; Yunita and Dodd, 2018).

Photobacterium, detected through amplicon sequencing, encompasses species that are naturally present in the marine environment with both symbiotic or pathogenic relationships with marine organisms (Labella et al., 2018). This bacterial genus was also detected by Reynisson et al. (2012) in fermented skate, where it was thought to play a key role in the fish flesh fermentation.

Although found in a limited number of samples, *Proteiniclasticum* and *Anaerobacillus* were also detected. The former genus includes anaerobic bacteria (e.g., *Proteiniclasticum ruminis*) with proteolytic activity (Zhang et al., 2010), whereas the latter genus includes species that can grow in alkaline environments (e.g., *Anaerobacillus alkalilacustre*) (Zavarzina et al., 2009).

The low occurrence of *Staphylococcus* suggests a possible contamination during the processing of *hákarl*, even though Ji et al. (2017) suggested a possible role of this genus in the bacterial amino acid metabolism occurring during the fermentation of the fish *Siniperca chuatsi*, together with *Acinetobacter*. Of note is that although found as a minority

490 species in the *hákarl* samples analyzed in the present study, Magnússon and Gudbjörnsdottir (1984) reported
491 *Acinetobacter* as one of the fermentation-driving microorganisms during the production, along with *Lactobacillus*.
492 Regarding *Ochrobactrum*, it is noteworthy that members belonging to this halophilic genus have recently been isolated
493 by Jamal and Pugazhendi (2018) in Red Sea saline water and sediments.
494 *Erysipelothrix* was sporadically detected in the analyzed *hákarl* samples through metagenomic sequencing.
495 Interestingly, species of this genus have already been detected in soils collected from the Ross Sea region of Antarctica,
496 which is characterized by extreme low temperatures and high water salinity (Aislabie et al., 2008).
497 *Listeria* was found as minority OTU by amplicon sequencing; notwithstanding, no viable cells belonging to the
498 pathogenic species *L. monocytogenes* were found in any of the analyzed *hákarl* samples in accordance with the results
499 reported by Jang et al. (2017) in fermented skate. It is noteworthy that in seafood from cold environments *L.*
500 *monocytogenes* represents a foodborne pathogen of increasing public health and food safety concern (Elbashir et al.,
501 2018; Jami et al., 2014).
502 Although the bacterial biota in *hákarl* has already tentatively been studied by Magnússon and Gudbjörnsdottir back in
503 1984, to our knowledge, the present study represents the first attempt to gain insight into the fungal biota present in this
504 fermented fish product.
505 The assessment of the diversity of yeast communities present in the *hákarl* samples revealed the occurrence of 4 yeast
506 genera: *Debaryomyces*, *Candida*, *Saccharomyces*, *Torulospora*, which are commonly found in several traditional
507 fermented beverages and food products, including fermented fish (Tamang et al., 2016), along with two other genera,
508 *Yamadazyma* and *Sporobolomyces.* The occurrence of sequences affiliated to *Debaryomyces* in all of the *hákarl*
509 samples suggests that this genus may be the main organism responsible for the late stages of *hákarl* fermentation. This
510 yeast, retrieved from the skin and inside the intestines of fresh fish by Andlid et al. (1995) and Gatesoupe (2007), was
511 also reported to be able to grow at extremely high salt concentrations and low water activity (a_w) (Asefa et al., 2009;
512 Viljoen and Greyling, 1995), characteristics of the ripened *hákarl*.
513 Our findings are consistent with previous reports showing the occurrence of this genus in salted and traditionally
514 fermented fish from Thailand and Ghana (Paludan-Muller et al., 2002; Sanni et al., 2002). The yeasts of the genus
515 *Debaryomyces* might positively contribute to the development of the sensory qualities of fermented fish, as they are
516 known to occur in cheeses and dry-cured meat products. More specifically, in cheeses with high salt content, *D.*
517 *hansenii* was found to predominate, being responsible for the acceleration of lipolysis and proteolysis (Andrade et al.,
518 2009).
519 Several sequences affiliated with different species from the genus *Candida* were retrieved in almost all the *hákarl*
520 samples. In particular, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, and *C. zeylanoides* were identified. Our results agree

with a previous report showing the dominance of the genus *Candida* in ripened *Suan yu*, a Chinese traditional fermented fish (Zang et al., 2018b) and a study demonstrating the presence of *C. zeylanoides* and *C. tropicalis* in a salted and fermented traditional fish called "*adjuevan*", which is produced in Ivory Coast (Clementine et al., 2012).

Candida was reported to produce more flavoring substances than other yeasts by metabolizing branched-chain amino acids (BCAAs) through the Ehrlich pathway (O'Toole, 1997). In particular, *C. tropicalis* was also isolated from *Burukutu*, a Nigerian traditional fermented beer, and it was shown to produce protease, phytase, lipase and esterase enzymes (Ogunremi et al., 2015). These last two enzymes improve the aromatic profile of fermented foods by increasing their free fatty acid content, which are precursors to the formation of different aromatic compounds (Arroyo-Lopez et al., 2012).

Three sequences affiliated with *Saccharomyces* were retrieved from the nine *hákarl* samples, confirming the presence of such yeasts in fermented fish (Clementine et al., 2012; Zang et al. 2018a).

Close relatives of *Yamadazyma* and *Sporobolomyces* were also detected in the H9 and H10 *hákarl* samples, respectively. These two yeast genera were not previously recovered from fermented fish products, although *Sporobolomyces* is a marine yeast commonly found in deep-sea waters (Kutty and Philip, 2008).

As for the occurrence of filamentous fungi, sequences affiliated with the genera *Alternaria*, *Cladosporium*, *Phoma/Epicoccum*, and *Moristroma* were retrieved from the analyzed *hákarl* samples. Among such fungal genera, *Cladosporium* was previously found during the fermentation of the Chinese traditional fermented fish *Suan yu* (Zang et al. 2018). The presence of filamentous fungi, such as *Aspergillus*, *Penicillium* and *Mucor*, was also reported from some Japanese fermented fish products, *i.e.*, *Katsuobushi* and *Narezushi*, where they produced enzymes such as amylase, protease, and lipase, which are important for the improvement of the nutritional and functional traits of fermented goods (Fukuda et al., 2014).

Conclusions

Overall, the combination of culture-dependent and -independent methods allowed major and minor microbial species harbored by the ready-to-eat *hákarl* samples to be detected. The culture-dependent approach provided insight into the viable microbial species, whereas the culture-independent methods were pivotal in preventing the possible underestimation of bacterial diversity caused by culturing biases or the presence of microbial cells in the "viable but non culturable" (VBNC) state. It is noteworthy that although amplicon sequencing was essential for detecting major and minor components of the bacterial biota, the use of PCR-DGGE led to the identification of both bacterial and fungal

populations at the species level, thus contributing to the development of a first overview of the microbiota occurring in this poorly studied food product.

Based on our results, *hákarl* revealed a complex and heterogeneous biodiversity. The bacterial community was characterized by species well adapted to alkaline and saline environments; the dominant genus was *Tissierella*, followed by *Oceanobacillus*, *Pseudomonas* and *Abyssivirga*. Moreover, based on the presence of *Pseudomonas* in the analyzed samples, a role of this bacterial genus in the detoxification of TMAO or TMA in shark meat during fermentation may be hypothesized. The fungal community was mainly represented by *Debaryomyces*, *Candida* and, to a lesser extent, *Saccharomyces* species, which through interactions with the bacterial community might play key roles in the late stages of *hákarl* fermentation, especially contributing to the development of the sensory qualities of the end product. Further studies are needed to establish the roles and the viabilities of the detected microbial species occurring during shark fermentation, as well as their interactions and relationships with the physical-chemical and rheological parameters of *hákarl*. Moreover, the occurrence of spore-forming bacteria should also be evaluated since the presence of these microorganisms has already been reported by different authors in fresh or fermented fish products (Metcalf et al., 2011, Reynisson et al.,2012). It is noteworthy that, among spore forming bacteria, *Clostridium botulinum* type E spores and toxins (produced even at low temperatures) can commonly be found in seafood (Elbashir et al., 2018; Iwamoto et al., 2010), thus representing a serious health threat for the consumers.

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806 **FIGURE LEGENDS**

807

808 **Fig. 1** Ready-to-eat *hákarl*

809

810 **Fig. 2.** Affiliation of the sequences retrieved from DGGE gel fragments (marked in Supplementary Figure 2) with the
811 existing sequences of the partial D1/D2 region of the large sub-unit rRNA gene.

812

813 Phylogenetic analysis was inferred by using the Maximum Likelihood method based on the kimura 2-parameter model.
814 Bootstrap (1000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA6. The
815 sequences from the database are indicated by their accession numbers. The DNA sequences retrieved in this work are
816 indicated by their corresponding band number and their accession number.

817

818 **Fig. 3.** Relative abundance (%) of yeast and filamentous fungal genera detected in each *hákarl* sample.

819

820 For each sample the percentage was calculated as follows: the number of fragments referring to a genus divided by their
821 total number.

822

823 **Fig. 4** PCA based on the OTU abundance of the *hákarl* grouped as a function of the samples.

824

825 The first component (horizontal) accounts for the 40.66% of the variance and the second component (vertical) accounts
826 for the 22.53 %

827

828 **Fig. 5** OTU network summarizing the relationships between taxa and samples.

829

830 Only OTUs occurring at 0.2% in at least 2 samples are shown. The abundances of OTUs in the two biological replicate
831 were averaged. The sizes of the OTUs were made proportional to weighted degree (i.e., for OTUs, this measures the
832 total occurrence of an OTU in the whole data set) using a power spline. OTUs and samples are connected with a line
833 (i.e., edge) to a sample node, and its thickness is made proportional to the abundance of an OTU in the connected
834 sample.

Table 1 Results of pH and viable counting (log cfu per gram) of bacteria and eumycetes in ready-to-eat *hákarl* samples

Sample	pH	Total mesophilic aerobes	Presumtive mesophilic lactobacilli	Presumptive mesophilic lactococci	Pseuodomonadaceae	Enterobacteriaceae	Eumycetes
H1	8.07±0.06	3.61±0.02	<1	4.51±0.04	<1	<1	<1
H2	8.23±0.01	5.71±0.09	1.60±0.43	4.39±0.01	1.54±0.34	<1	<1
H3	8.20±0.01	1.24±0.34	<1	4.27±0.05	<1	<1	<1
H4	8.09±0.01	1.30±0.00	<1	3.84±0.08	1.59±0.16	<1	<1
H5	8.37±0.01	2.40±0.02	<1	3.20±0.17	1.30±0.00	<1	<1
H6	8.53±0.04	2.01±0.15	<1	3.22±0.31	1.15±0.21	<1	<1
H7	8.41±0.00	1.15±0.21	<1	1.95±0.07	1.00±0.00	<1	<1
H8	8.41±0.01	1.00±0.00	<1	2.39±0.06	<1	<1	<1
H9	8.46±0.01	2.24±0.09	<1	3.30±0.08	1.24±0.34	<1	<1
H10	8.76±0.00	2.01±0.15	<1	4.04±0.15	<1	<1	<1

Values are expressed as means ± standard deviation

Table 2. Sequencing results of the bands excised from the DGGE gel obtained from the amplified fragments of bacterial DNA extracted directly from the pooled *hákarl* samples

Sample	Band[a]	Identification	% Identity[b]	Most closely related GeneBank sequence
H1	1	*Tissierella creatinophila*	98%	NR_037028
	2	*Anaerosalibacter massiliensis*	97%	NR_144694
	3	*Anaerosalibacter* sp.	93%	LT598565
	4	*Tissierella creatinophila*	97%	NR_117377
	5	*Tissierella creatinophila*	95%	NR_117377
H2	6	*Anaerosalibacter massiliensis*	97%	NR_144694
	7	*Tissierella creatinophila*	97%	NR_117377
H3	8	*Tissierella creatinophila*	85%	NR_117377
H4	9	*Tissierella creatinophila*	98%	NR_117377
H5	10	*Murdochiella massiliensis*	97%	NR_148568
	11	*Tissierella creatinophila*	98%	NR_117377
H6	12	*Tissierella creatinophila*	96%	NR_117377
	13	*Sporanaerobacter acetigenes*	97%	NR_117381
	14	*Tissierella creatinophila*	95%	NR_117377
H7	15	*Tissierella creatinophila*	97%	NR_117377
	16	*Tissierella creatinophila*	94%	NR_117377
H8	17	*Tissierella creatinophila*	97%	NR_117377
H9	18	*Tissierella creatinophila*	97%	NR_117377
	19	*Tissierella creatinophila*	90%	NR_117377
H10	20	*Tissierella creatinophila*	97%	NR_117377
	21	*Pontibacillus marinus*	97%	LT992038
	22	*Anaerosalibacter massiliensis*	97%	NR_144694
	23	*Anaerosalibacter massiliensis*	93%	NR_144694

[a] Bands are numbered as indicated in Supplementary Figure 1

[b] Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database.

Table 3 Identification of yeasts and filamentous fungi occurring in the *hákarl* by sequencing the fragments obtained from DGGE profiles.

DGGE fragments[a]	Identification	Identity (%)[b]	Most closely related GeneBank sequence
H2-1	*Candida tropicalis* CBS:2320	99%	KY106851.1
H2-2	*Torulaspora delbrueckii* CBS 1146	100%	NG 058413.1
	Torulaspora pretoriensis CBS 11124		KY109883.1
	Torulaspora quercuum CBS 11403		NG 058413.1
H2-3	*Cladosporium tenuissimum* QCC:M024/17	100%	KY781762.1
H3-4	*Candida parapsilosis* CBS:2915	100%	Y102317.1
H3-5	*Debaryomyces hansenii* CBS:11096	100%	KY107560.1
	Debaryomyces prosopidis JCM 9913		NG_055701.1
	Debaryomyces subglobosus JCM 1989		NG_055699.1
	Debaryomyces fabryi CBS:4373		KY107483.1
H3-6	*Saccharomyces cerevisiae* CBS:7961	100%	KY109313.1
H4-7	*Debaryomyces hansenii* CBS:11096	100%	KY107560.1
	Debaryomyces prosopidis JCM 9913		NG_055701.1
	Debaryomyces subglobosus JCM 1989		NG_055699.1
	Debaryomyces fabryi CBS:4373		KY107483.1
H6-8	*Candida zeylanoides* CBS:947	100%	KY106918.1
H6-9	*Alternaria sp strain* QCC/M011/17	99%	KY744118.1
H6-10	*Debaryomyces hansenii* CBS:11096	100%	KY107560.1
	Debaryomyces prosopidis JCM 9913		NG_055701.1
	Debaryomyces subglobosus JCM 1989		NG_055699.1
	Debaryomyces fabryi CBS:4373		KY107483.1
H6-11	*Alternaria* sp. isolate 1A1	100%	MF379649.1
H6-12	*Epicoccum sp CN018*	100%	KX954392.1
	Phoma herbarum JN0408		MG004796.1
H7-13	*Moristroma quercinum* BN1678	98%	AY254051.1
H7-14	*Candida zeylanoides* CBS:947	100%	KY106918.1
H7-16	*Debaryomyces hansenii* CBS:11096	100%	KY107560.1
	Debaryomyces prosopidis JCM 9913		NG_055701.1
	Debaryomyces subglobosus JCM 1989		NG_055699.1
	Debaryomyces fabryi CBS:4373		KY107483.1
H7-17	*Candida glabrata* CBS:859	99%	KY106478.1
H8-18	*Candida parapsilosis* CBS:2193	99%	KY102320.1
H9-19	*Yamadazyma mexicana* CBS 7066	97%	NG_058439.1
H9-20	*Debaryomyces hansenii* CBS:11096	100%	KY107560.1
	Debaryomyces prosopidis JCM 9913		NG_055701.1
	Debaryomyces subglobosus JCM 1989		NG_055699.1
	Debaryomyces fabryi CBS:4373		KY107483.1
H9-21	*Saccharomyces cerevisiae* CBS:7961	100%	KY109313.1
H10-22	*Debaryomyces hansenii* CBS:11096	100%	KY107560.1
	Debaryomyces prosopidis JCM 9913		NG_055701.1
	Debaryomyces subglobosus JCM 1989		NG_055699.1
	Debaryomyces fabryi CBS:4373		KY107483.1
H10-23	*Sporobolomyces salmoneus CBS:488*	100%	KY109767.1
	Sporobolomyces roseus OL10		KF273854.1
H10-24	*Saccharomyces cerevisiae* CBS:7961	100%	KY109313.1

[a] Bands are numbered as indicated in Supplementary Figure 2

[b] Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database.

Table 4 Incidence of the major taxonomic groups detected by 16S amplicon target sequencing. Only OTUs with an incidence above 0.2% in at least 2 biological replicate were averaged.

OTU	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Abyssivirga	0.19	2.88	2.92	5.73	3.56	5.52	2.93	6.88	4.81	4.41
Acinetobacter	0.00	0.04	0.90	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Alkalibacterium	0.06	0.15	0.10	0.24	0.13	0.19	0.18	0.18	0.21	0.23
Anaerobacillus	0.00	0.91	0.01	0.00	0.40	0.06	0.00	0.02	0.00	0.00
Erysipelothrix	0.05	0.15	0.23	0.20	0.13	0.13	0.05	0.11	0.13	0.02
Lactococcus	0.46	0.14	0.05	0.26	0.07	0.11	0.09	0.06	0.29	0.67
Listeria	0.00	0.29	0.00	0.00	0.16	0.05	0.00	0.02	0.01	0.00
Oceanobacillus	43.69	1.84	2.17	3.76	0.71	3.32	5.08	1.52	4.05	1.15
Ochrobactrum	0.00	0.57	0.00	0.00	0.27	0.06	0.00	0.01	0.01	0.00
Photobacterium	0.01	0.67	0.07	0.10	0.14	1.00	0.35	0.10	0.20	0.20
Proteiniclasticum	0.00	0.14	0.43	0.35	0.24	0.03	0.00	0.08	0.13	0.00
Pseudomonas	42.99	0.29	0.31	0.25	0.65	8.24	13.04	4.65	2.57	10.96
Staphylococcus	0.00	0.17	0.00	0.00	2.71	0.00	0.00	0.00	0.00	0.00
Tissierella	12.37	87.77	92.56	88.66	90.42	80.75	77.82	86.07	87.09	82.11

OTU Operational Taxonomic Units

Fig. 1

Fig. 2

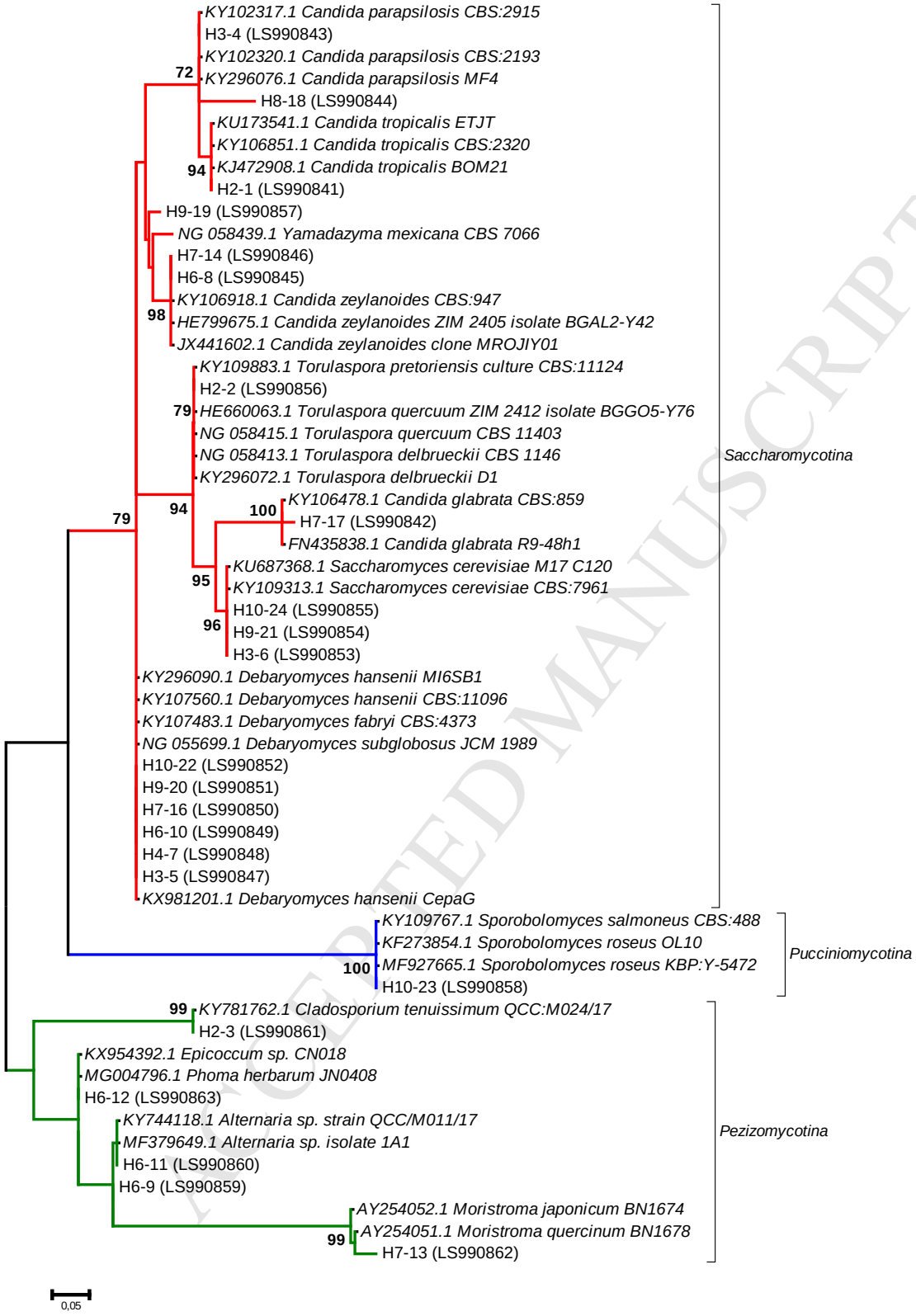


Fig. 3

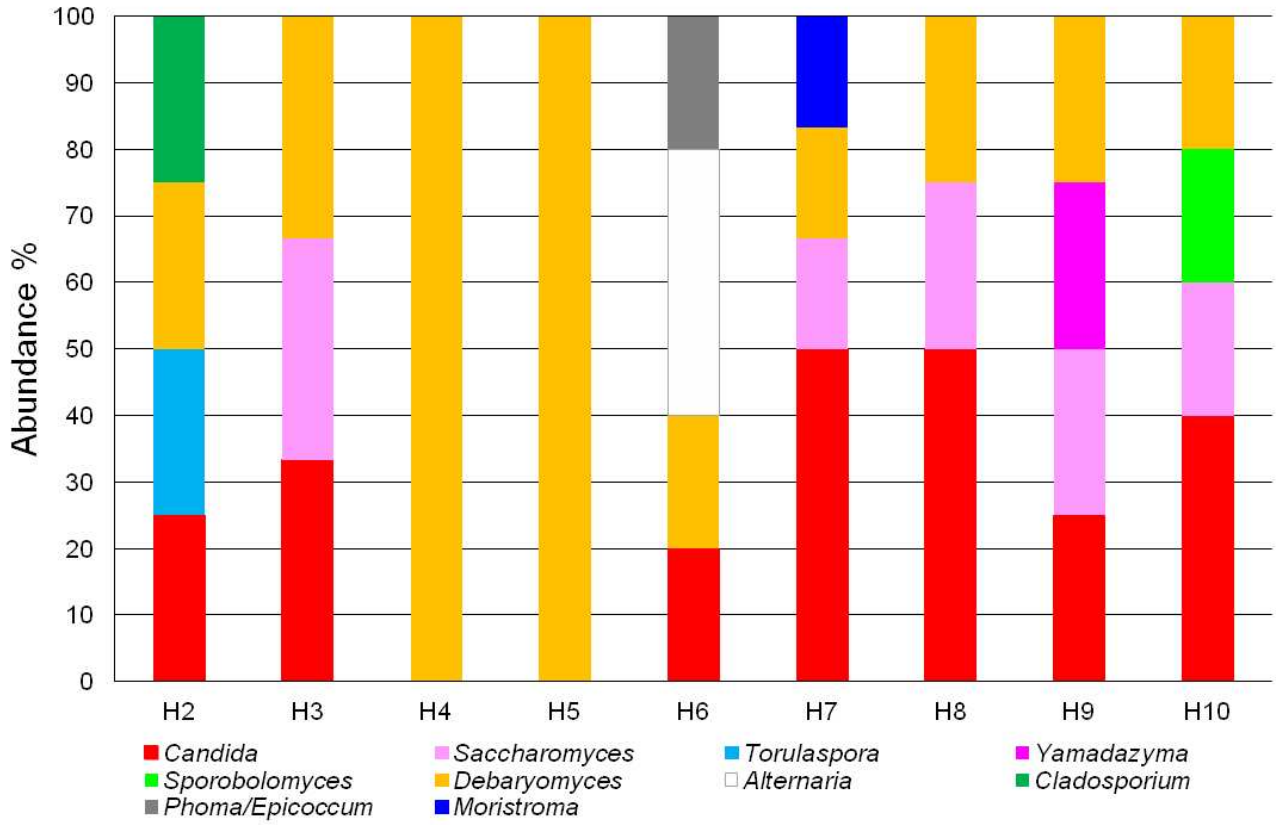


Fig. 4

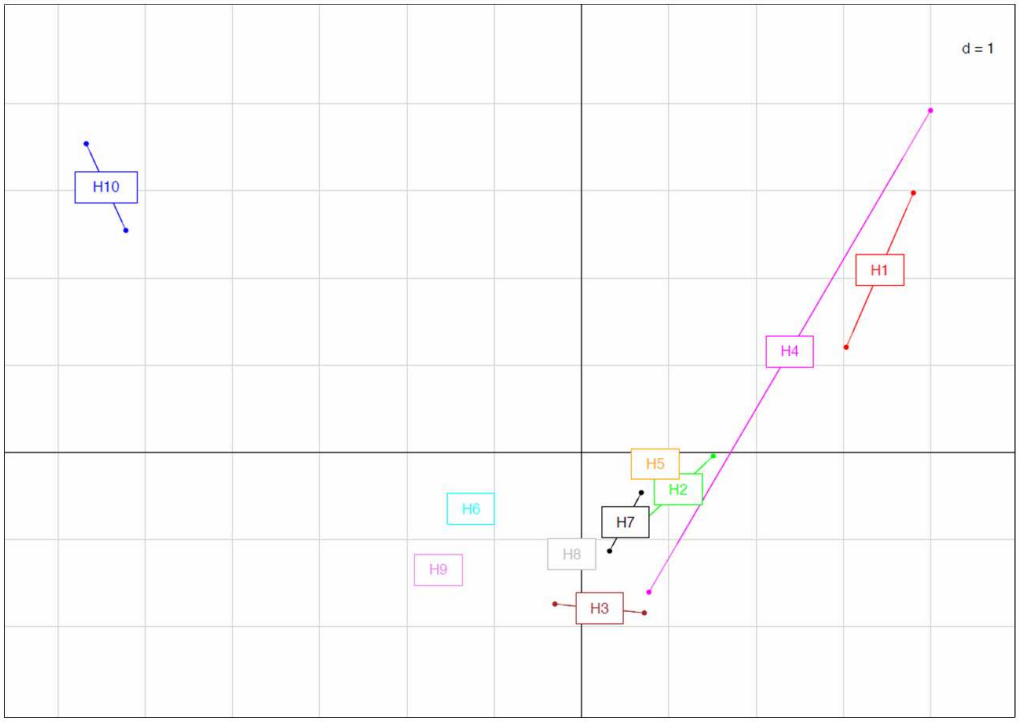
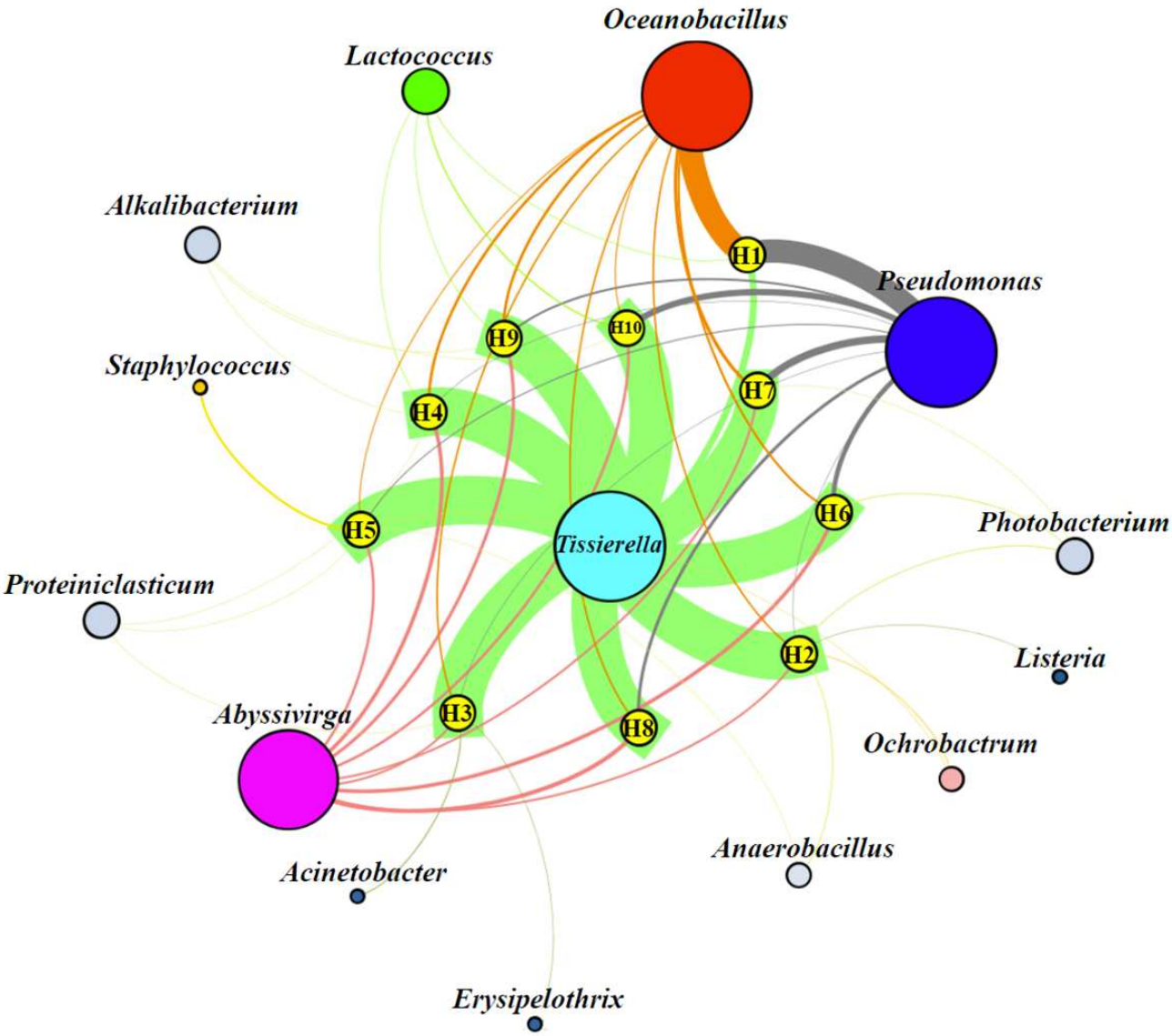


Fig. 5


Oceanobacillus
Lactococcus
Alkalibacterium
H1
Pseudomonas
H9
H10
Staphylococcus
H7
H4
H5
Tissierella
H6
Photobacterium
Proteiniclasticum
H2
Listeria
Abyssivirga
H3
H8
Ochrobactrum
Anaerobacillus
Acinetobacter
Erysipelothrix

Highlights

The microbiota of *hákarl* was studied using a polyphasic approach

Both metagenomic sequencing and PCR-DGGE highlighted the dominance of *Tissierella* sp.

The contribution of *Pseudomonas* in the detoxification of shark meat was hypothesized

The fungal community was mainly represented by *Debaryomyces* and *Candida* species

Real-time PCR showed the absence STEC and *Pseudomonas aeruginosa* in all the samples